

EXHIBIT 9

Antiviral Chemistry & Chemotherapy 14:205-215

ME-609: a treatment for recurrent herpes simplex virus infections

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Studies in conventional murine models of HSV infection use immunologically naive animals. These models thus mimic primary infections rather than recurrent infections in humans. We have, therefore, used a newly developed mouse model that more closely mimics recurrent HSV infection in humans. In this model, the mice are infected, and zosteriform HSV-1 infection develops in the presence of a primed immune response using adoptive transfer of immunity (ATI) as we have described previously. Using the ATI mouse model, it has been shown that a more beneficial therapy for recurrent mucocutaneous HSV infection could be achieved by controlling both the viral replication and the inflammatory response to the virus. Topical treatment was initiated in this model at the time of first occurrence of symptoms and was

given three times daily for 4 days. Topical treatment with ME-609 (which contains 5% acyclovir and 1% hydrocortisone) in the ATI mouse model was substantially more efficacious than 5% Zovirax® cream, 1% hydrocortisone or no treatment, respectively. The beneficial properties of ME-609 were also found to be superior to those of Zovirax® cream when tested in the standard guinea pig model, representing a primary HSV infection. ME-609 represents a novel treatment principle of recurrent HSV infections and the present paper summarizes the preclinical and early clinical experience of ME-609.

Key words: HSV-1, animal models, acyclovir, hydrocortisone, immune response.

Introduction

Primary infection with herpes simplex virus (HSV) usually occurs as a labial infection in childhood or as a genital infection in early adulthood, and is characterized by viral replication usually lasting approximately 10–14 days. The immune response is not fully developed until at least 7 days after infection. The lesions heal soon after the virus is cleared by the immune response (Alenius *et al.*, 1982; Spruance *et al.*, 1977; Spruance, 1992). Since healing of the lesions is a result of viral clearance, treatment with antiviral drugs is able to shorten the clinical episode by curtailing viral replication faster. Thus, treatment of primary genital HSV infections with oral or intravenous administration of antiviral drugs, such as acyclovir, reduces the period of viral

shedding and gives a significant improvement in clinical parameters such as pain and time to healing (Richards *et al.*, 1983). This pattern of extended viral replication that resolves as the immune response develops is also shown in traditional animal models of primary HSV infection, such as primary cutaneous infection of guinea pigs with HSV (Alenius *et al.*, 1982). In such animal models of primary HSV infection, treatment with antiviral drugs substantially reduces the duration of viral replication and thus the clinical episode (Alenius *et al.*, 1982; McKeough & Spruance, 2001; Poli *et al.*, 2001).

During primary infection, the virus enters the nerve endings and is transported in the axons towards the

ganglion, where the virus remains latent within the ganglionic neurons for the rest of the individual's life. At a later date, the virus may be reactivated and may travel back down the nerve to the skin, where it replicates producing a clinical episode of reactivated HSV infection. The recurrent disease differs from the primary episode in that the virus is typically cleared much more rapidly (within 3 days or less) due to the immediate immune response, which is already primed after previous episodes (Spruance *et al.*, 1977; Spruance, 1992). However, although in recurrent infection the immune response is much quicker and more effective, it is also the cause of most of the clinical symptoms of pain, redness, swelling and tenderness, through the inflammatory response to the virus. There is strong evidence that cell-mediated immune responses contribute significantly to the tissue damage and lesion development associated with HSV infection. Thus, although the virus is cleared more rapidly, the symptoms usually remain for about a week after the time at which virus can no longer be isolated, and the lesion typically takes 7–10 days to heal (Spruance, 1992; Spruance *et al.*, 1977). Consequently, treatment of recurrent HSV infection with exclusively antiviral drugs has little effect on the clinical parameters of disease, even though the duration of viral shedding may be shortened (Corey *et al.*, 1982a,b; Raborn *et al.*, 2002; Reichman *et al.*, 1983; Spruance & Crumpacker, 1982; Spruance *et al.*, 2002; Spruance *et al.*, 1997).

The characteristics of recurrent HSV infection, i.e. short period of viral replication and excessive inflammatory response to the virus, are not reflected in presently available animal models. We have, therefore, developed a new animal model, the adoptive transfer of immunity (ATI) mouse model, of HSV infection using zosteriform spread of virus in combination with ATI. This model mimics both the clinical parameters of reactivated HSV infection and the effects of antiviral treatment (Awan *et al.*, 1998 a,b). Using this model, we have developed a new approach for the treatment of recurrent HSV infection in that both viral replication is curtailed and the inflammatory response controlled. This is achieved by combining an antiviral compound with an agent with immunomodulatory properties in a topical formulation. Such a topical combination would have the dual properties of both inhibiting viral replication and controlling the inflammatory response and promoting healing. A topical formulation of 5% acyclovir and 1% hydrocortisone (ME-609), aiming to have these properties, has been developed by Medivir AB and AstraZeneca.

We have evaluated this new concept in cell culture experiments, skin penetration experiments and animal models of primary HSV infection as well as in our new ATI mouse model, which mimics recurrent HSV infections. Recently, Phase I and II clinical studies have been performed that confirm the ability of the ATI mouse

model to predict efficacy against recurrent HSV disease (Evans *et al.*, 2002).

Materials and methods

Cell culture studies

Cells and virus. Human embryonic lung (HEL) fibroblasts (kindly provided by the Swedish Institute for Infectious Disease Control, Solna, Sweden) and African green monkey kidney (Vero) cells (ATCC, Rockville, Maryland, USA) were propagated in Earl's Modified Eagle's Medium (GibcoBRL Laboratories, Inc., Gaithersburg, Md., USA) supplemented with 10% foetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C in 5% CO₂. The C42 P2 strain of HSV-1 (a clinical isolate obtained from the Swedish Institute for Infectious Disease Control, Solna, Sweden) was grown and titrated in Vero cells by plaque assay.

HSV-1 (strain SC16) was originally isolated from a clinical case of herpes labialis (Hill *et al.*, 1975). Virus was grown in baby hamster kidney cells (BHK-21) following established procedures (Field *et al.*, 1995) and stored at -70°C.

Cell culture procedure. HEL fibroblasts (4×10⁵ cells) were seeded into 12-well plates and grown for 3 days at 37°C in 5% CO₂. Confluent cells were inoculated with the C42 p2 strain of HSV-1 in serum-free medium at a multiplicity of infection of 0.001. After 45 min of adsorption at 37°C in 5% CO₂, serum supplemented medium containing acyclovir in the range of 0–10 µM in combination with hydrocortisone in the range of 0–100 µM was added. Cells were incubated for an additional 48 h at 37°C in 5% CO₂ and then supernatants were removed and frozen at -80°C.

Vero cells (2×10⁵ cells) were plated and grown in 24-well plates. At confluence, cells were infected with the viral supernatants eluted in serum-free medium in series of dilutions from 10⁻² and to 10⁻⁶. After 1 h of adsorption fresh medium supplemented with 10% foetal bovine serum and 2 mg/ml human immunoglobulins (Beriglobin, Centeon Pharma GmbH, Marburg, Germany) was added. The infection proceeded for 3 days at 37°C in 5% CO₂, after which cells were stained with crystal violet and plaques were counted. The result was shown as plaque forming units (PFU)/ml.

Formulations

ME-609 is a cream formulation containing 5% acyclovir and 1% hydrocortisone. All excipients are well-known and widely used in topical products. All formulations were prepared at CCS AB, Borlänge, Sweden. Commercially

available Zovirax® cream, containing 5% acyclovir, was repacked to preserve blinding whenever appropriate. Commercially available 1% hydrocortisone cream was obtained from CCS AB, Borlänge, Sweden.

Guinea pig experiments

The backs of the Dunkin-Harley guinea pigs were plucked, shaved and depilated with Opilca® (GlaxoSmithKline Consumer Healthcare, Copenhagen, Denmark) as previously described (Alenius & Oberg, 1978). Twenty microlitres of HSV-1 strain C42 (10^6 PFU/ml) was applied to four areas on each animal on day 0 using a spring-loaded vaccination instrument according to previously described procedures (Hubler *et al.*, 1974). Two areas on each animal were treated with topical placebo and served as controls. The other two areas were treated with different topical formulations containing antiviral compounds. Treatment was started at 24 or 48 h after infection depending on experimental design (see figure legends for details) and continued for 3 or 4 days. All non-commercial formulations containing active ingredients used in the experiments were blinded to the investigator. In a subsequent experiment (Figure 1b), commercially available Zovirax® cream was repacked into similar tubes by third party and the study was thus blinded. The inoculated areas were assessed for clinical signs of HSV lesions once daily throughout the experiments until healing. All scoring was performed in a blinded fashion (Table 1). The score system was adopted from previously published procedures (Alenius & Oberg, 1978).

ATI mouse model

The model itself and some results using the model have previously been published (Awan *et al.*, 1998a,b).

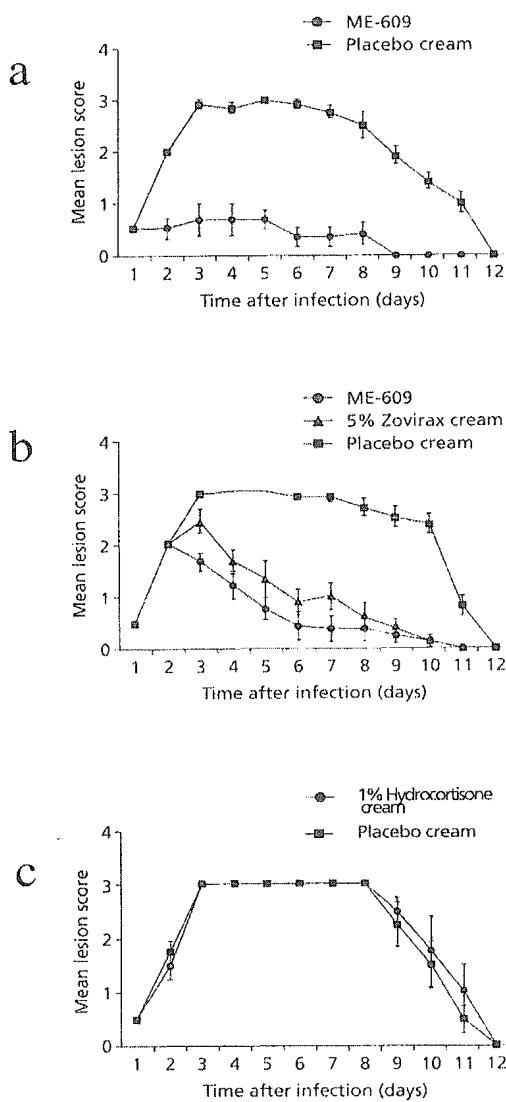
Inoculation of mice

Inoculation was performed as previously described (Blyth *et al.*, 1984). Briefly virus suspension (2×10^5 PFU) was placed on the skin of female Balb/C mice, weighing 16–18 g, on the right side of the ventral surface of the neck, then 8–10 superficial light strokes were made by a hypodermic needle in a criss-cross pattern to produce the scarification.

Adoptive transfer of immunity (ATI). A group of immune donors were prepared by inoculation of HSV-1 (10^5 PFU) into the skin of both left and right ears of female Balb/C mice. The cervical lymph nodes were removed at 7 days after infection and a suspension of lymph node cells was prepared. Recipient mice were given 3.6×10^6 live immune cells via the coccygeal vein. The recipient mice had been infected 2 days previously with HSV-1 by neck scarification (see above).

Experimental design. The animals were divided into three treatment groups and one untreated control group.

Figure 1. Effect of ME-609 on lesion score in a primary HSV-1 infection in guinea pigs



Three or four areas on six to nine guinea pigs were infected on day 0 with HSV-1, as described in 'Materials and methods'. One area on each animal was treated topically with ME-609 from day 1 (Figure 1a and 1c) or day 2 (Figure 1b) for 4 days. One area on each animal was treated with 5% Zovirax® cream while the remaining two areas were treated with placebo. Treatments were applied twice daily (Figure 1a) or four times daily (Figure 1b) for 4 days. The lesion score was assessed daily until healing. The average lesions score and standard error of each day is shown. The effect of 1% hydrocortisone cream applied twice daily for 3 days was compared with placebo in eight areas using similar procedure (Figure 1c). Statistical analysis showed that ME-609 treatment resulted in significant improvement over placebo in both Figure 1a ($P=0.014$) and Figure 1b ($P<0.001$) study settings. The difference between ME-609 and 5% Zovirax® treatment (Figure 1b) was not statistically significant.

Table 1. Lesion scoring system

Lesion score	Appearance of inoculated skin
0.5	Erythematous and slightly oedematous
1	Erythema and one or two small vesicles
2	Erythema and numerous small vesicles
3	Numerous large vesicles; if in close juxtaposition, coalesced
3	Vesicles dried, large crusts
2	Circa 50% crusts fallen off
1	Circa 10% of the crusts remaining
0	Uninfected or healed area, no crusts or vesicles; trauma from inoculation or traces from infection can be present

All of these animals were given ATI. Each study group consisted of 10 animals for observation (zoster score and ear thickness increase). In addition, 16 animals were included in each group for titration of virus. Four animals were sacrificed for virus titration on days 5, 7, 9 and 13 post infection (p.i.), respectively.

Application of study creams. The ear pinnae of the mice were treated topically with study creams three times a day at 8 h intervals i.e. 08:00, 16:00 and midnight. The topical treatment was started on day 4 at 08:00 p.i. and continued to day 7 at midnight p.i. All creams were packed in identical containers and coded and the study was thus blinded.

Clinical disease assessments. The thickness of the ear pinna, ipsilateral to the infection, was measured daily using an Engineers' micrometer (Field *et al.*, 1979). The development of lesions with respect to zoster score on the neck (primary site) and pinna (zosteriform spread) was monitored visually using a magnifying glass. The zosteriform lesions were scored according to a scale previously described (Nagafuchi *et al.*, 1979). The following zoster scores were used; 0 unchanged ear, 1 isolated zosteriform lesions, 2–4 describe the ulceration of confluent zosteriform lesions from mild to severe.

Virus titration. At various times, mice were euthanized and each mouse's ear ipsilateral to the infection site was removed and homogenized. Following topical treatment, there is a possibility that the antiviral drug may be present on the ear samples obtained and this may affect the virus isolation. The skin was, therefore, thoroughly washed before homogenization and virus titration. The theoretical maximum concentration of acyclovir in the titration was then not high enough to affect the plaque counts. As a further precaution, 50 µM of thymidine was added to the growth medium to decrease any possible remaining anti-

viral activity of acyclovir. Acyclovir is activated by phosphorylation by virus specific thymidine kinase and addition of thymidine to the growth medium blocks activation of any remaining acyclovir (Harmenbergs, 1983). Virus was titrated as previously described (Field *et al.*, 1995).

In vitro skin penetration

The backs of the Dunkin-Harley guinea pigs were plucked, shaved and depilated with Opilca® as previously described (Alenius & Oberg, 1978). Two days after depilation the animals were sacrificed and full thickness skin was removed and frozen in -70°C.

The subcutaneous fat was removed by blunt dissection and the skin samples were applied to a two-chamber diffusion cells system as previously described (Aulton, 1988). The upper chamber was open to facilitate cream application to the skin surface (surface area 0.93 cm²). Both formulations studied in the experiment were blinded. The receiving chamber contained Ringer solution and samples were taken at various times following application of creams to the skin surface.

The acyclovir content of the samples from the receiving chamber was analysed by HPLC with UV-detection using a mobile phase of 0.05 M (NH₄)₂PO₄ buffer at pH 7.00 and 15% methanol. The column used was a 150×2.1 mm C18 Zorbax 5 µm particle size reversed phase column. The mobile phase was delivered at 0.2 ml/min and detection was at 254 nm. The retention time of acyclovir in this system was approximately 2.7 min.

Statistical methods

Statistical analyses used for comparisons between treatments (ear thickness and zoster score using the ATI mouse model) were based on Kruskal-Wallis for testing all treatments against each other. A significant *P*-value indicated that at least one treatment differs systematically from the others, and was thus followed by *post hoc* pair wise comparisons using the Mann-Whitney U test. In the analyses of the zoster score, the individually observed difference between day 4 and day 9 was known since all observations at day 4 were equal to 0. For the analysis of ear thickness, the observations were dependent but not individually identified. The number of degrees of freedom was still limited to the number of independent observations (Altman, 1991).

The viral growth (log transformed) in the cell studies was evaluated by a one way ANOVA for each of the ACV concentrations 0, 0.1 and 1 µM. An overall significant result was followed by *post hoc* ANOVA multiple comparisons.

An overall ANOVA with both ACV and HC included as factors was not feasible due to an uncontrollable heterogeneity of the variance structure.

The cumulative scores, which were proportional to a possible area under the curve, in the guinea pig

experiments were used for comparison between treatments. The analysis was made conservative by considering the most positive result of the two placebo scores. As all guinea pigs have all treatments the Friedman ANOVA for ranks was used, followed by *post hoc* tests using Friedman multiple comparisons for ranks.

In vitro skin penetrations profiles: ME-609 was compared with 5% acyclovir cream by first estimating the intensity over time for each guinea pig (the slope after linearization of the process). The mean intensities for the two groups were then compared using a t-test (the estimated intensities were found to reasonably follow a normal distribution).

The virus titres (log transformed) for untreated and 1% hydrocortisone cream-treated mice were compared over time (5, 7, 9 and 13 days) using a two factor Mixed Model ANOVA with substance and time as factors and mouse as replicates with substance and time. The Mixed Model analysis was used due to a non-homogenous variance structure over time.

Results

Cell culture studies

After infection with the clinical HSV-1 isolate C42 p2, HEL cells were treated for 48 h with 0–10 μ M acyclovir in the presence of 0–100 μ M hydrocortisone (Figure 2). Treatment with acyclovir significantly ($P<0.001$) inhibited viral multiplication in accordance with previous experience (Harmenberg *et al.*, 1980). The addition of hydrocortisone (up to 100 μ M) did not alter the inhibition by acyclovir *in vitro*. Statistical analysis showed that hydrocortisone treatment alone in the range of 0.1–100 μ M did not influence the viral growth.

Guinea pig experiments

The antiviral activity of ME-609 was tested using the primary HSV-1 infection guinea pig model. Animals treated with placebo formulation developed skin lesions within 2 days of infection. The lesions healed around day 10–12 p.i.

ME-609 showed a pronounced beneficial effect in this model both when treatment was initiated 24 hours and 48 h after infection (Figure 1a, $P=0.014$ and 1b, $P<0.001$). The average lesion score was below 1 at all times when treatment was initiated at 24 h and most areas were only erythematous and slightly oedematous. The lesion development was thus interrupted by ME-609 treatment.

When treatment was delayed to 48 h after infection to assess the effect on developed lesions, treatment with ME-609 was at least as effective as treatment with 5% Zovirax[®] cream even though superiority could not be shown with statistical methods (Figure 1b). The comparison between ME-609 and Zovirax[®] cream was performed in repeated experiments with similar results. ME-609 tended to be superior at all times tested (data not shown). Commercially available 1% hydrocortisone cream did not, as expected, reduce lesion score in this model (Figure 1c). However, the small size of the data set precluded statistical analysis.

Virus titres in the skin of the animals were assessed at the peak of the virus infection (day 5) and immediately after the viral shedding period. Similar low virus titres were obtained from Zovirax[®] cream and ME-609-treated areas (data not shown). No prolongation of the viral shedding period was seen. Furthermore, all treatments were safe and no treatment-related adverse effects were noted.

ATI mouse model experiments

Ear thickness increase and zoster score. Treatment with topical ME-609 showed beneficial effects in terms of both ear thickness increase and zoster score (Tables 2 and 3). Furthermore, the results indicated that ME-609 treatment was more effective than the other treatments. Two different parameters were calculated: the average of the zoster score and ear thickness increase on day 9 p.i. and the average cumulative zoster score and ear thickness increase. Day 9 p.i. was selected as an appropriate time when full effect of the different treatments was expected. Day 9 p.i. is also a time when the clinical signs (ear thickness increase and

Table 2. Effects of different treatments on ear thickness increase using the ATI mouse model

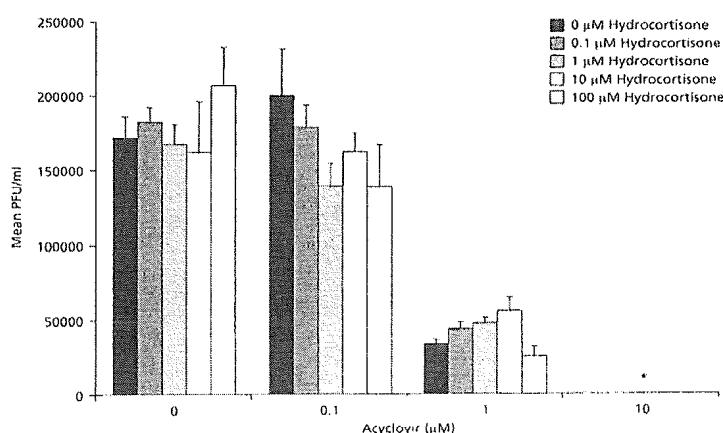
	Average increase in ear thickness \pm SE (mm)*	% of control	Average day 4–15 increase in cumulative ear thickness (mm)†	% of control
ME-609	0.15 \pm 0.03‡	34%	1.04	14%
Zovirax [®] cream	0.48 \pm 0.08	110%	3.99	100%
1% hydrocortisone cream	0.23 \pm 0.03	52%	2.74	69%
Untreated control	0.43 \pm 0.05		3.99	

* Average day 9 p.i. increase in ear thickness (mm) compared with day 4 baseline.

† Increases from day 4 p.i. (baseline) calculated for day 4–10, 12, 13 and 15 p.i. In addition, extrapolated values from day 11 and 14 p.i. The mice were not individually marked and standard error could therefore not be obtained.

‡ The ME-609-treated group was significantly better than the untreated control group ($P<0.001$), the 1% hydrocortisone cream-treated group ($P<0.04$) and the 5% Zovirax[®] cream group ($P<0.001$).

Figure 2. Effects of acyclovir and hydrocortisone on HSV-1 replication in HEL cells



Viral multiplication in HSV-1-infected HEL cells treated with a combination of increasing concentrations of acyclovir and hydrocortisone. Cells were infected at a multiplicity of infection of 0.001 and, after 45 min of absorption, fresh medium containing acyclovir and hydrocortisone was added. The infection proceeded for 48 h and then viral supernatants were frozen at -80°C before being titrated in Vero cells. Results shown as average with standard error. Statistical analysis showed that hydrocortisone treatment alone in the range of 0.1–100 μM of hydrocortisone did not influence the viral growth. *no virus detectable in any group

Table 3. Effects of different treatments on zoster score using the ATI mouse model

	Average zoster score \pm SE at day 9 p.i.	% of control	Average cumulative zoster score	% of control (day 4–15 p.i.)*
ME-609	2.0 \pm 0.2†	58%	15.3	60%
Zovirax® cream	2.4 \pm 0.3	70%	19.1	75%
1% hydrocortisone cream	2.8 \pm 0.2	80%	23.6	93%
Untreated control	3.4 \pm 0.3		25.3	

* Measurements summated for day 4–10, 12, 13 and 15 p.i. In addition, extrapolated values from day 11 and 14 p.i. The mice were not individually marked and standard error could therefore not be obtained.

† The ME-609 group was significantly better than the untreated control group ($P<0.001$), the 1% hydrocortisone group ($P<0.01$) while the difference from the 5% Zovirax® cream group was not statistically significant.

zoster score) are approaching their peak values. For ear thickness increase, the increase in value over day 4 p.i. (baseline value) was used in the calculations. The average cumulative zoster score and ear thickness increase as calculated in Tables 2 and 3 were used as the second parameter. Here again, the changes over baseline were used for the ear thickness increase calculations.

ME-609 was shown to improve both the day 9 p.i. ear thickness increase and the average cumulative ear thickness increase by 66% ($P<0.001$) and 86% in comparison to untreated control mice. Similarly, the average day 9 p.i. zoster score and the average cumulative zoster score were improved by ME-609 treatment by 42% ($P<0.001$) and 40% in comparison with untreated control mice, respectively. The ME-609 treatment was more efficacious

than treatment with Zovirax® cream ($P<0.001$) or 1% hydrocortisone cream ($P<0.04$) with respect to ear thickness increase. ME-609 treatment was better than hydrocortisone cream treatment with respect to zoster score ($P=0.01$), while the improvement vs. Zovirax® cream did not reach statistical significance in the present experiment.

Zovirax® cream did not show any effect on ear thickness increase. When measuring zoster score, Zovirax® cream showed a relatively good effect in comparison with untreated control animals (Tables 2 and 3). This effect was greater than previously seen in the ATI mouse model (unpublished data).

Hydrocortisone cream showed a substantial effect on ear thickness increase, as expected, while the effect on zoster score was limited (Tables 2 and 3). The variability (mea-

sured as standard error) of the results in the ATI mouse model at time points after the peak of infection is however substantial.

Virology data

Groups of four mice were euthanized on days 5, 7, 9 and 13 after infection and the virus titres of the ears were assessed by plaque assay. The results showed that both ME-609 and Zovirax® cream reduced the virus titres over time compared with untreated control mice at all time points in a similar fashion (Figure 3). Hydrocortisone treatment increased the viral shedding over time compared with the untreated control group ($P=0.04$) and low but measurable virus titres were also found on day 13 p.i.

Skin penetration

ME-609 and a 5% acyclovir cream formulation, similar to Zovirax® cream, were tested on full thickness guinea pig skin ($n=7-8$). In this experiment, application of ME-609 reached higher acyclovir concentrations in the receiving chamber than 5% acyclovir cream (Figure 4, $P=0.0004$).

Discussion

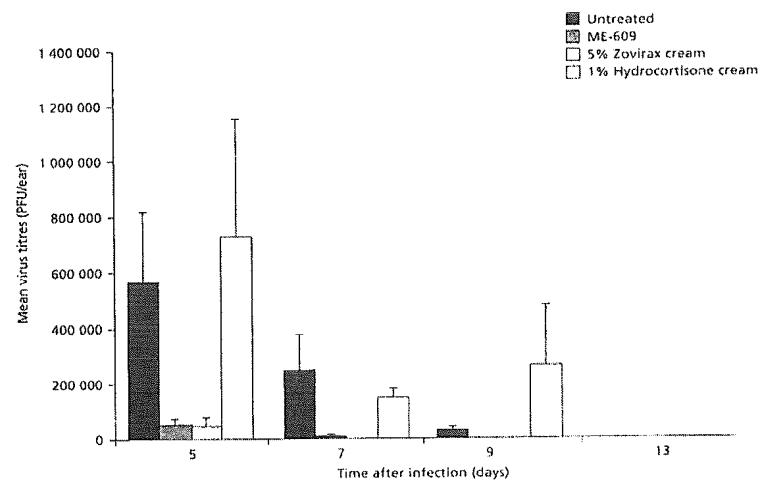
As stated in the introduction, primary infection models fail to mimic the clinical situation with respect to recurrent HSV infections. The ATI mouse model offers the possibility of representing the immunological as well as the virological pattern associated with recurrent HSV infections in man. The ATI mouse model is, however, labour-intensive

and cumbersome to use. In addition, it is a complex model with many different parameters that can contribute to experimental variation. It is, therefore, critical that all treatments and assessments are performed blinded as in the present study. The ATI mouse model can be used to assess proof-of-principle but it would be difficult to perform detailed assessments, for example dosing or dosing intervals. In addition, mice will lick their ears and oral exposure to the topical drugs cannot be excluded, even though a small amount of treatment is applied and the gastrointestinal uptake of acyclovir and hydrocortisone is limited. Finally, mice will scratch their infected ears, which could worsen the symptoms and increase experimental variation. This is especially true in the untreated groups or the groups receiving suboptimal treatment and at later time points, after the peak of infection.

Three parameters were used to assess the effectiveness of different formulations in the ATI mouse model. Zoster score is the traditional way to determine clinical progression of herpetic lesions in rodents. It is however a subjective parameter dependent on the experience of the investigator. The immune response and the subsequent inflammation induced by the virus infection of mouse ears cause increased ear thickness, a parameter that can readily be objectively measured by mechanical means. Virus titrations of the tissues also provide quantitative information.

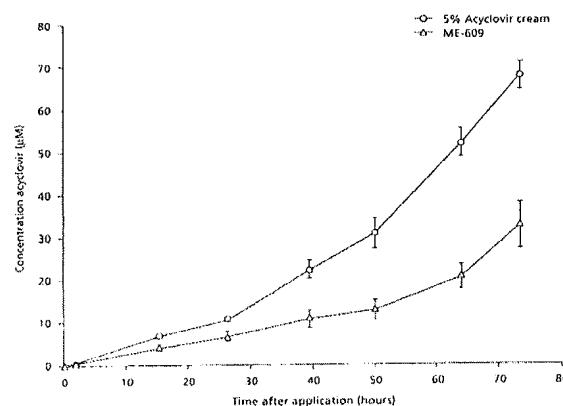
Previous experience with the ATI mouse model has been summarized in two published articles (Awan *et al.*, 1998a,b). We initially used the ATI mouse model to study antivirals in combination with a large number of substances

Figure 3. Viral titres in ATI mice infected with HSV-1 and given topical treatments



Mean virus titres and standard error in mouse ears measured at various times after infection. Groups of four animals were sacrificed at day 5, 7, 9 and 13 after infection. The virus titres of the ears were assessed as described in 'Materials and methods'. The virus titres were below the limits of detection (<20 PFU/ear) in the 5% Zovirax® group on day 7 and in both the ME-609 and the 5% Zovirax® group on day 9. On day 13 all groups were below the limits of detection except the 1% hydrocortisone cream group, which showed a mean virus titre of 100 PFU/ear. The hydrocortisone treated group showed statistically increased viral shedding over time compared with the untreated control group ($P=0.04$).

Figure 4. Penetration of acyclovir through guinea pig skin from topical ME-609 and acyclovir cream



Acyclovir penetration profiles of ME-609 and 5% acyclovir cream applied topically to full thickness guinea pig skin. ME-609 was applied to eight skin samples at time 0. Acyclovir cream was applied to seven skin samples. The concentration of acyclovir in the receiving chamber was measured at various time points shown as average with standard error. Application of ME-609 resulted in significantly higher acyclovir concentrations in the receiving chamber over time compared with 5% acyclovir cream treatment ($P=0.0004$).

with immunomodulatory properties including, e.g. glucocorticosteroids, non-steroidal anti-inflammatory drugs and local anaesthetics (unpublished). Combinations of antivirals with glucocorticosteroids were shown to be more beneficial than other combinations. We also found that the combination of antiviral drugs with hydrocortisone was more effective than combinations with other glucocorticosteroids (unpublished).

The most important result of the present study is the demonstration that the combination of antiviral compounds (in this case, acyclovir) with a mild immunomodulatory agent (hydrocortisone) is clearly beneficial with respect to the measured parameters. The control groups (acyclovir alone, hydrocortisone alone and untreated infected mice) all showed less effect on the measured parameters than the ME-609 treated group. As expected, the mice treated with Zovirax® cream did not show any beneficial effect with respect to ear thickness increase. However, the result suggested that Zovirax® cream had a somewhat better effect on zoster score than could be expected based on previous experience with foscarnet (Awan *et al.*, 1998a) and acyclovir (unpublished data). This could be explained by experimental variation as discussed above or by the fact that only 3.6×10^6 live immune cells were given as ATI to recipient mice in the present experiment which was less than the intended target of $1-2 \times 10^7$ cells. Both ME-609

and Zovirax® cream treatment reduced the virus titres compared to untreated control mice.

Hydrocortisone cream treatment had a good effect on ear thickness increase during the treatment period (days 4-7 p.i.). After the treatment period, the effect of hydrocortisone used alone was less pronounced compared to ME-609 treatment. Hydrocortisone alone had little effect on zoster score. In addition, hydrocortisone cream increased the period of viral shedding and low levels of virus could be detected until day 13 p.i. Similar prolongation of infectious virus in tissues has been shown in previous published (Awan *et al.*, 1998a) and unpublished studies. It is therefore tempting to speculate that the possible extended viral shedding period may cause the late increase in ear thickness seen in mice treated with only hydrocortisone cream or other corticosteroids (data not shown) (Awan *et al.*, 1998a). ME-609 treatment reduced both the amount and length of viral shedding as compared to untreated mice. This is in accordance with previous experiments using combinations of foscarnet and hydrocortisone (Awan *et al.*, 1998a) and combinations of acyclovir and hydrocortisone (unpublished).

In all groups (except the ME-609 group), the experimental variation as measured by the standard error was larger after the peak of infection (day 9-10 p.i.) compared with earlier time points. This could be due to the effectiveness of ME-609 resulting in less scratching of the ears by the mice in this group, presumably due to less itching. As discussed above, mice scratch their ears and this may contribute to the clinical picture at the later stage of infection.

ME-609 was also evaluated in the cutaneous guinea pig model using primary HSV-1 infection. This model is a well-documented primary infection model (Alenius, 1980; Alenius *et al.*, 1982; Alenius & Oberg, 1978) and generalization of results to recurrent infections is, as noted above, problematic. It does, however, confirm the acyclovir efficacy in primary infection and thus indicate sufficient skin penetration of acyclovir from the ME-609 vehicle.

In the cutaneous infection of guinea pigs with HSV-1, treatment with hydrocortisone alone had no effect upon lesion development or duration in comparison with placebo; for example, hydrocortisone cream neither worsened the severity of the lesions nor prolonged their duration. In addition, hydrocortisone cream did not influence viral shedding of the lesions (data not shown). This was expected, as in this model of primary infection the guinea pigs are seronegative for HSV at infection and only develop immunocompetence gradually during the duration of the experiment. As discussed below, the cell culture studies also failed to show stimulation of viral growth by steroids.

ME-609 was more effective in reducing the severity and duration of lesions in the guinea pig model than treatment with acyclovir alone. The beneficial effect of ME-609 was

also seen when treatment was delayed until 48 h p.i.. This was unexpected, given that hydrocortisone alone had no effect and given that this is a primary infection in seronegative animals. One possible explanation is that hydrocortisone has several other effects in addition to the inhibition of inflammation, such as vasoconstriction. The vasoconstrictive effects of hydrocortisone may lead to locally higher concentrations of acyclovir in the skin, resulting in an improved antiviral effect in the guinea pig model. The penetration of acyclovir through the skin has been linked to the antiviral effect as measured in guinea pigs (Freeman *et al.*, 1986; Freeman & Spruance, 1986; Spruance *et al.*, 1986; Spruance *et al.*, 1984). In addition, acyclovir has a problematic penetrational profile when used in a topical formulation and the first commercially available acyclovir formulation, an ointment, has been connected with both poor penetration and poor efficacy in animals and clinical trials (Spruance & Crumpacker, 1982; Spruance *et al.*, 1986; Spruance *et al.*, 1982). An improved acyclovir cream formulation was later developed and this formulation showed better acyclovir penetrational properties as well as improved efficacy (Spruance *et al.*, 1986; Spruance *et al.*, 2002). Acyclovir in the ME-609 formulation penetrates guinea pig skin readily and even better than from the commercially available acyclovir cream as shown in *in vitro* skin penetration experiments described in Figure 4. This result showed that the ME-609 cream formulation delivers acyclovir through intact skin efficiently and is at least as good as the improved acyclovir cream formulation that is available in certain countries.

Glucocorticosteroids have been suggested to potentially stimulate viral growth (and growth of other microorganisms) through two different mechanisms: direct stimulation of viral growth and indirect alteration (inhibition) of the immune system. This concern has primarily been addressed with respect to primary virus infections where the immune system has not previously been exposed to the infectious agent rather than reactivated infections. Results from the primary infection guinea pig model, as well from the ATI mouse model, showed that viral shedding profiles after ME-609 and Zovirax® cream treatment were similar. In addition, the *in vitro* studies of HSV-1-infected cells treated with acyclovir in combination with hydrocortisone showed no change in effect on the viral replication level compared to cells treated with acyclovir alone. In addition, the virus levels of the ME-609-treated animals were below those of the control animal in both animal models. Clearly, the ME-609 concept was safe in both models tested.

Treatment with hydrocortisone alone increased duration of viral shedding in the ATI mouse model representing recurrent infections and measurable virus titres were found 13 days after infection while no virus could be found in the untreated control group beyond day 9. The virus titres in

the present study were similar in the untreated and the hydrocortisone cream treated-group at the assessments on days 5 and 7. These results confirmed previous published studies showing that hydrocortisone treatment increased the duration of viral shedding up to day 15 after infection (Awan *et al.*, 1998a). The present study failed to confirm the 100–1000 fold increase in viral titres on days 5 and 7 by hydrocortisone cream treatment previously found (Awan *et al.*, 1998a). The reason for this discrepancy is presently not known. No enhancement of viral growth was however seen in HSV-1-infected cell culture. Nor did treatment with hydrocortisone alone influence the development of lesions in the primary HSV-1 infection model in guinea pigs. This indicates that the enhanced viral growth observed in the ATI mouse model is more likely a result of the altered immune response than direct stimulation of viral replication even though a glucocorticoid response element has been found in the HSV-1 genome (Hardwicke & Schaffer, 1995). Published data on cell culture studies with glucocorticoid treatment of HSV-infected cells are also conflicting. Depending on the cell line used different published studies have shown increases, decreases or no changes in the viral replication level (Hardwicke & Schaffer, 1995; Hardwicke & Schaffer, 1997; Nishiyama & Rapp, 1979; Notter & Docherty, 1978). Clearly more work is needed to resolve this issue.

In conclusion, we have used a novel animal model using a combination of zosteriform infection with adoptive transfer of immunity (ATI) that is believed to better mimic recurrent HSV infections than the currently available models (Awan *et al.*, 1998b). The ATI has previously been shown to increase inflammation (measured by ear thickness) and zoster score with around 50% and to decrease the viral shedding time from 7 to 4 days in parallel to findings in clinical trials (Awan *et al.*, 1998b). In the ATI mouse model, antiviral compounds, such as foscarnet and acyclovir, used alone only show modest beneficial effects, which is reflected in experience from clinical trials. The ME-609 combination of acyclovir and hydrocortisone was more efficacious in reducing the clinical parameters in this animal model. This is probably due to reduction of viral replication, by acyclovir, in combination with modulation of the immune response known to cause the clinical symptom, by hydrocortisone.

The present preclinical studies suggest that ME-609, a new combination of acyclovir and hydrocortisone, may be beneficial in the treatment of recurrent HSV infections in man and thus constitutes a new treatment principle in this disease. The local tolerance of ME-609 was good when tested in animals and in humans. The predicted clinical usefulness of the ME-609 combination principle has been confirmed in two independent recently published clinical trials (Evans *et al.*, 2002; Spruance & McKeough, 2000).

Both studies induced recurrent herpes labialis by exposing individuals to ultraviolet light. Both studies suggested that early treatment with a combination of an antiviral and an immunomodulator decreased the number of patients developing ulcerative HSV lesions by roughly a third compared with treatment with an antiviral alone or placebo. The ulcerative recurrent HSV lesions that did develop, notwithstanding combination treatment, were smaller and healed faster compared with control patients. These clinical data validate the usefulness of the novel ATI mouse model in predicting therapeutic benefit on recurrent HSV disease.

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— Received 10 November 2002; accepted 2 June 2003 —